STUDIES ON THE REGULATION OF *STR3* IN *Saccharomyces cerevisiae*

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BS-MS dual degree in Science

By

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To the

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Certificate of Examination

This is to certify that dissertation titled **" STUDIES ON THE REGULATION OF *STR3* IN *Saccharomyces cerevisiae* "**submitted by Ms. Bindia Chawla (MS12045) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Shravan K.Mishra	Dr. Kavita Babu	Prof. Anand Kumar Bachhawat (Supervisor)

Dated: April 21,2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. A.K. Bachhawat at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Bindia Chawla

Date: April 21,2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Anand Kumar Bachhawat

(Supervisor)

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ABBREVIATIONS

Weights and measures

%	Percent
μmol, nmoles, mmoles,	micromole, nanomoles, millimoles,
°C	Degree centigrade
bp, kb	Base pair, kilobase
kDa	Kilodalton
O.D.	Optical density
Psi	Pounds per square inch
rpm	Revolutions per minute
RT	Room temperature
sec, min, h	Second, minute, hour,
μg, mg, g	microgram, milligram, gram
μl, ml, L	Microliter, milliliter, liter,
μM, mM, M,	micromolar, millimolar, molar

Symbols

~	Approximately
=	Equal to
α	Alpha
β	Beta
γ	Gamma
Δ	Delta

Techniques

PCR	Polymerase Chain Reaction

Chemicals

Amp	Ampicillin
ATP	Adenosine Triphosphate
dNTPs	2’-deoxyadenosine 5’-triphosphate
EDTA	Ethylenediamine-tetra-acetic acid
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HCl	Hydrogen Choride
PEG	Poly Ethylene Glycol

Miscellaneous

YEASTRACT	*Yeast Search for Transcriptional Regulators and Tracking*
DNA	Deoxyribonucleic acid
EUROSCARF	European *S. cerevisiae* archive for functional Analysis
GFP	Green Fluorescent Protein
LB	Luria Bertani
NCBI	*National Center for Biotechnology Information*
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
TE	Tris chloride and EDTA
WT	Wild-type

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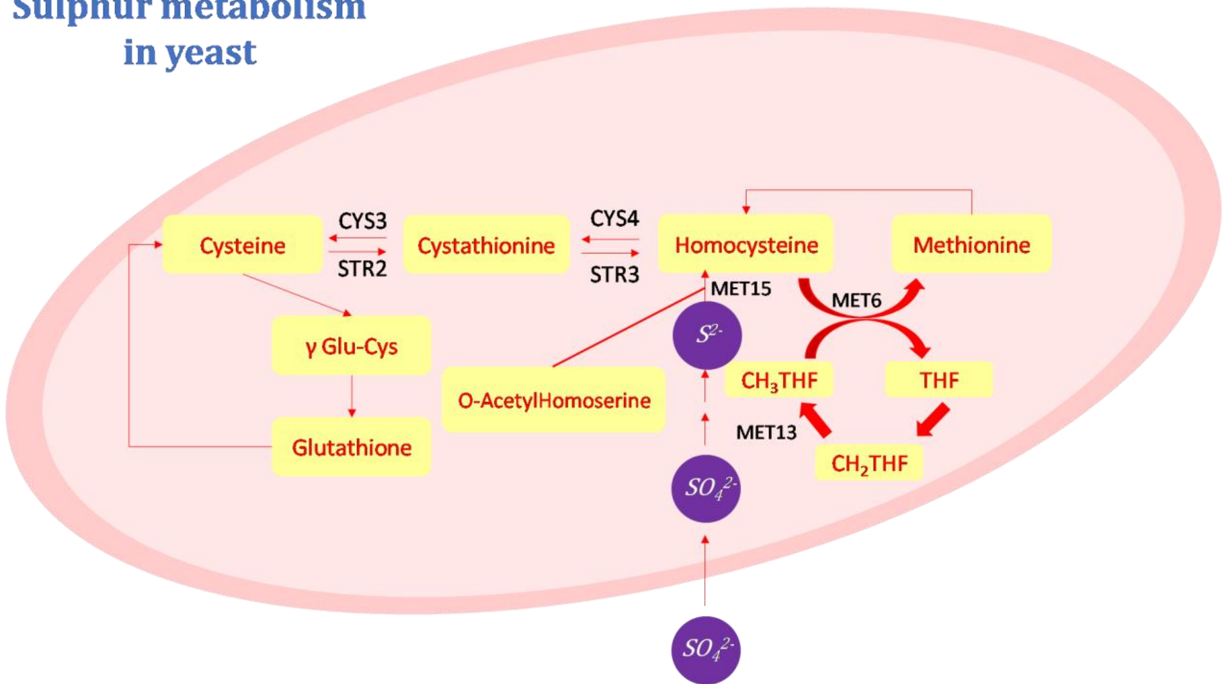
Abstract

The *STR3* gene of *Saccharomyces cerevisiae* encodes the enzyme cystathionine beta lyase. This enzyme of the sulphur pathway converts cystathionine to homocysteine. *STR3* is known to be under sulphur regulation, but recent studies have suggested that the regulation of *STR3* might be more complex. Phylogenetic analysis of the *STR3* promoter was carried out to initially identify conserved motifs in the promoter. This was followed up by expression analysis of the *STR3* promoter using STR3-GFP reporter fusions. The role of the conserved motifs were investigated by deletion analysis and also by examining expression in different transcription factor deletion backgrounds. This analysis suggested a role for *GCN4* in achieving full sulphur derepression. We also investigated the roles of the three *MET31/MET32* motifs present in the *STR3* promoter but could not find a role, as seen by the mutation of individual motifs. *STR3* also did not appear to be upregulated by intracellular cystine based on the reporter gene fusion studies, nor was it affected by the *MET12* gene product. A two hybrid analysis of Met12p –Str3p interaction revealed that the two proteins do not appear to physically interact.

Chapter 1

Introduction and Review of Literature



1 Introduction

1.1 Importance of Sulphur

Sulphur is one of the essential elements required for the growth of living organisms. Sulphur is a building block of many important organic molecules like enzymes, vitamins, proteins and also of two amino acids- methionine and cysteine. Sulphur is also a constituent of glutathione which is the most important antioxidant molecule providing protection to cellular components from reactive oxygen species. Sulphur is present in many compounds with oxidative states ranging from -2 to +6. *Saccharomyces cerevisiae* has evolved the ability to use a wide range of inorganic and organic sulphur sources.

1.2 Sulphur Assimilation in *Saccharomyces cerevisiae*

In the yeast *Saccharomyces cerevisiae*, uptake of inorganic sulphur compounds like sulphates occurs through the high affinity sulphate transporters *SUL1* and *SUL2*. The intracellular sulphate is then converted to sulphide through a series of steps. Homocysteine synthase (*MET15*) then helps in the conversion of sulphide to homocysteine. Homocysteine pools are then used for the production of methionine, cysteine and glutathione (Thomas and SurdinKerjan, 1997). In reverse trans-sulphuration pathway, homocysteine first converts to cystathionine by cystathionine β synthase (*STR4*) and subsequently to cysteine by cystathionine γ lyase (*CYS3*). These cysteine pools are further used for protein synthesis and production of glutathione. From cysteine, homocysteine can be produced via two successive reactions of trans-sulphuration pathway. In the first reaction cysteine is converted to cystathionine. This reaction is catalyzed by cystathionine γ synthase (*STR2*). The second step converts cystathionine to homocysteine by cystathionine β lyase (*STR3*).

Methionine is produced from homocysteine by means of homocysteine methyltransferase (*MET6*).Methylene tetrahydrofolate reductase (*MET13*) reduces methylene tetrahydrofolate to methyl tetrahydrofolate which later donates a methyl group to homocysteine for the conversion to methionine.

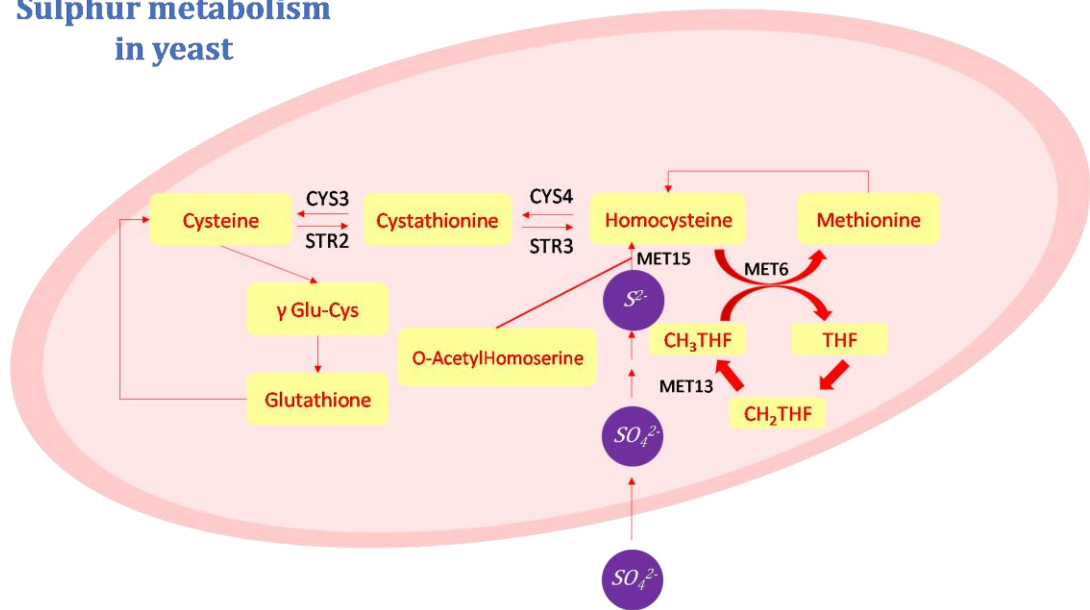


Figure1.1: Schematic representation of sulphur metabolism in *Saccharomyces cerevisiae*

1.3 Sulphur regulation in *Saccharomyces cerevisiae*

MET4,*MET28*,*CBF1*,*MET30*,*MET31* and *MET32* are the genes which codes for principle transcription factors involved in sulphur regulation of *Saccharomyces cerevisiae.*(Thomas and SurdinKerjan, 1997).Met4p is the main transcriptional activator of sulphur metabolic network, whereas Met28p,Cbf1p,Met31p and Met32p act as adaptors for binding of Met4p to its target promoters (Lee et al., 2010). CACGTG and AAACTGTGGC are the two main cis-regulatory elements identified to be involved in regulation of sulphur pathway genes (Lee et al., 2010; Thomas and SurdinKerjan, 1997).

Met4p is a basic leucine zipper domain (bZIP) containing factor which is the main transcription activator of sulphur pathway genes. Met4p itself lacks intrinsic DNA binding ability and depends on DNA-binding cofactor proteins- Cbf1p, Met31p and Met32p to reach its target promoters (Lee et al., 2010; Thomas and SurdinKerjan, 1997).

Met28p is also a basic leucine zipper domain (bZIP) containing factor which itself lacks any intrinsic transcription activation function but is known to be positive regulator of transcription of many *MET* genes. Met28p is known to stabilize binding of Cbf1p-Met4p

and Met31p/Met32p-Met4p complexes to DNA (Lee et al., 2010; Thomas and SurdinKerjan, 1997)

Cbf1p is a basic helix-loop-helix (bHLH) domain containing factor and this domain helps Cbf1p to bind to TCACGTG sequence (Thomas and SurdinKerjan, 1997).

Met30p functions as a transcriptional inhibitor of Met4p under repressive conditions. Under repressive sulphur conditions, Met30p leads to polyubiquitylation of Met4p further leading to its proteolysis (Lee et al., 2010; Thomas and SurdinKerjan, 1997).

Met31p and Mte32p are two zinc-finger containing proteins which like Cbf1 provides DNA binding ability to Met4p. Both of them help in transcriptional activation but in Met4p dependent manner and binds to the consensus sequence AAACTGTGGC (Lee et al., 2010; Thomas and SurdinKerjan, 1997).

1.4 Transcriptional regulation of *STR3*

Cystathionine, an intermediate in sulphur metabolic pathway is converted to homocysteine by cystathionine β lyase (*STR3*) and to cysteine by cystathionine γ lyase (*CYS3*). Both *STR3* and *CYS3* are shown to be upregulated during sulphur limitation conditions (Boer et al., 2003; Tai et al., 2005). Increased expression of *STR3* has been reported in *Saccharomyces cerevisiae* upon exposure to excess of cysteine along with cystathionine accumulation in the cell (Deshpande et al., 2017). Microarray profiles have also shown upregulation of *STR3* in sulphur limiting and *MET4* hyperactivation conditions (Lee et al., 2010). Also, strong upregulation of *STR3* gene has been shown by giving high levels of polyamines (spermidine) in media conditions.(Chattopadhyay et al., 2009)

1.5 Link between *MET12* and *STR3*

Methylene tetrahydrofolate reductase (*MET13*) reduces methylene tetrahydrofolate to methyl tetrahydrofolate which remethylates homocysteine to methionine, a step catalyzed by methionine synthase (*MET6*). *met13*Δ and *met13*Δ*met15*Δ are methionine auxotrophs. *MET12* is proposed to be a homolog of *MET13* based on sequence similarity yet a

*met12*Δ strain grows well on all organic as well as inorganic sulphur sources unlike *met13*Δ. Surprisingly, *met12*Δ*met15*Δ strain was unable to utilize GSH, cysteine as well as cystathionine as organic sulphur sources but was able to grow at homocysteine suggesting a block at cystathionine to homocysteine conversion step catalyzed by cystathionine β lyase (*STR3*) [Bhatia M. unpublished].

Aim and objective of the present study

The principal goal of this study was to identify possible novel elements in *STR3* regulation.

More specifically, we have listed out the following objectives:

1. A detailed *in-silico* analysis of the *STR3* promoter and identification of any motifs or transcription factor binding sites
2. Creation of an STR3-GFP reporter fusion and using this to investigate the *STR3* under a variety of nutrient and genetic backgrounds
3. To carry out a deletion analysis and a mutational analysis of critical elements of the *STR3* promoter
4. To investigate possible interactions at the transcriptional level or the protein level between *STR3* and *MET12*

Chapter 2

Materials and Methods

SECTION A: MATERIALS

2.1. CHEMICALS AND REAGENTS

All the chemicals used were obtained from commercial sources and were of analytical grade. Media components, fine chemicals and reagents were purchased from Sigma Aldrich, (St. Louis, USA), HiMedia, (Mumbai, India), Merck. India Ltd (Mumbai, India), USB Corporation (Ohio, USA) or Difco, USA. Oligonucleotides (primers) were designed using Snapgene software and were purchased from Integrated DNA Technologies (IDT). Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, *Vent* DNA polymerase, *Taq* DNA polymerase and other modifying enzymes), their buffers and dNTPs were purchased from New England Biolabs Inc, (Beverly, MA, USA). Gel-extraction kits and plasmid miniprep columns were obtained from Bioneer. Methionine, GSH and cysteine were obtained from Sigma-Aldrich, USA.

2.2. STRAINS AND PLASMIDS

Escherichia coli DH5α was used as the cloning host. The genotype for the *E. coli* strain and the yeast strains used in the study are given in Table 1. The list of various plasmids used in this study is given in Table 2.

Table 1: List of bacterial and yeast strains used in the study

Strain	Genotype	Source
***Escherichia coli* strain**		
DH5ά	*F' gyr A(Nal) recA1 relA endA1 thi-1 Hsd 17(rk-mk+) gln V44 deoR Δ(lacZYA-argF) U169 [ϕ80dΔ(lacZ) M15]*	Lab strain
Yeast strain of *S. cerevisiae*		
ABC 733	*BY 4741. MATa his3Δ-1 leu2-Δ0 met15Δ0 ura3Δ0*	Lab strain
ABC 734	*BY 4742. MATα his3Δ-1 leu2-Δ0 lys2Δ0 ura3Δ0*	Lab strain
ABC 1849	*MATα ura3Δ his3Δ leuΔ lys2Δ YJR060w (CBF1)::KANMX2*	Lab strain
ABC1097	*MATα ura3Δ his3Δ leuΔ lys2Δ YPL038w(MET31)::KANMX2*	Lab strain
ABC 1098	*MATα ura3Δ his3Δ leuΔ lys2Δ YDR253c (MET32)::KANMX2)*	Lab strain
ABC 1539	*MATα ura3Δ his3Δ leuΔ lys2Δ YIR017c (MET28)::KANMX2*	Lab strain
ABC 1520	*MATa his3Δ-1 leu2-Δ0 met15Δ0 ura3Δ0 YKR099W (BAS1)::KANMX4*	Lab strain
ABC 1528	*MATa his3Δ-1 leu2-Δ0 met15Δ0 ura3Δ0 YEL099C (GCN4)::KANMX4*	Lab strain
ABC 2973	*MATa his3Δ-1 leu2-Δ0 met15Δ0 ura3Δ0 YEL031W(HAC1)::KANMX4*	Lab strain
ABC 1517	*MATa his3Δ-1 leu2-Δ0 met15Δ0 ura3Δ0 YML007W(YAP1)::KANMX4*	Lab strain
ABC 4961	*MATα ura3Δ his3Δ leuΔ lys2Δ YDR207C(UME6)::KANMX2*	Lab strain
ABC 1116	*MATα ura3Δ his3Δ leuΔ lys2Δ YJR095w(URE2)::KANMX2*	Lab strain
ABC 1094	*MATα ura3Δ his3Δ leuΔ lys2Δ YER 040w (GLN3)::KANMX2*	Lab strain

ABC 993	***EGY48 MATα trp1 his3 ura3 leu2::6LexAOp-LEU2***	**Lab strain**

Table 2: List of Plasmids used in the study

Clone No.	Plasmid Name	Description
ABE 2097	pRS426-MET17-GFP	1.6 Kb MET2 GFP excised from ABE 1435
ABE 2099	pRS426-GFP	Cloned by 1 kb BamI-XhoI excision of GFP from ABE 2096 and cloned into ABE 259
ABE 5326	pRS426-STR3p600-GFP	STR3 promoter of length 600bp amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Confirmed by restriction digestion.
ABE 5327	pRS426-STR3p441-GFP	STR3 promoter of length 441bp amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Confirmed by restriction digestion.
ABE 5328	pRS426-STR3p395-GFP	STR3 promoter of length 395bp amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Confirmed by restriction digestion.
ABE 5329	pRS426-STR3p363-GFP	STR3 promoter of length 363bp amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Confirmed by restriction digestion.
ABE 5330	pRS426-STR3p288-GFP	STR3 promoter of length 288bp amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099).Confirmed by restriction digestion.
ABE 5331	pRS426-STR3p256-GFP	STR3 promoter of length 256bp amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Confirmed by restriction digestion.
ABE 5391	p426-STR3p600-GFP with MET31/32 1st motif bases mutated	STR3 promoter of length 600bp with -411 and -412 C, A mutated to TT amplified from p426-STR3p600-GFP (ABE5326) and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Clone confirmed by restriction digestion and sequencing
ABE 5392	p426-STR3p600-GFP	STR3 promoter of length 600bp with -358 and -359 T,G mutated

	with MET31/32 2nd motif 2 bases mutated	to AA amlified from p426-STR3p600-GFP (ABE5326) and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Clone confirmed by restriction digestion and sequencing
ABE 5393	p426TEF-STR3p600-GFP with MET31/32 3rd motif 2 bases mutated	STR3 promoter of length 600bp with -249 and -250 T,G mutated to AA amlified from p426-STR3p600-GFP (ABE5326) and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Clone confirmed by restriction digestion and sequencing
ABE 5446	p426-STR3p600-GFP with MET31/32 1st motif 5 bases mutated	STR3 promoter of length 600bp with -410 to -414bases ACACC mutated to TTTTT amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Clone confirmed by restriction digestion.
ABE 5447	p426-STR3p600-GFP with MET31/32 2nd motif 5 bases mutated	STR3 promoter of length 600bp with -356 to -360 bases GGTGT mutated to AAAAA amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Clone confirmed by restriction digestion.
ABE 5448	p426-STR3p600-GFP with MET31/32 3rd motif 5 bases mutated	STR3 promoter of length 600bp with -247 to -251 bases tGTGG mutated to AAAAA amplified from genomic DNA and cloned between BamHI and XhoI sites of pRS426-GFP (ABE2099). Clone confirmed by restriction digestion
ABE 720	pEG202	A HIS3+ plasmid for making LexA fusions (i.e. the bait plasmid)
ABE 1006	pJG4-5	Target fusion plasmid TRP, 2 micron, Ampr, GAL1 promoter expresses B42-HA-tagged fusion proteins.
ABE 721	pSH18-34	A plasmid carrying URA3+, LacZ reporter with LexA binding site. 2uM with Ampr, URA3. Needed to test for LexA fusion. It containes 8 LexA operators upstream of Gal1-LacZ.
ABE 723	pSH17-4	A plasmid with HIS3+ as a selectable marker. A positive control for transcriptional activation. This plasmid expresses LexA-GAL4 fusion that activates transcription.
ABE 724	pRFHM1	A plasmid with HIS3 as selectable marker. Needed as a negative control for transcriptional activation. This plasmid expresses LexA-bicoid fusion.

ABE 5451	MET12-pEG202	MET12 gene digested with BamHI and XhoI sites of ABE 2781 and subcloned in bait plasmid pEG202 between BamHI and XhoI sites. Clone confirmed by restriction digestion.
ABE 5453	STR3-pEG202	STR3 gene digested with BamHI and XhoI sites of ABE 4102 and subcloned in bait plasmid pEG202 between BamHI and XhoI sites. Clone confirmed by restriction digestion.
ABE 5499	MET12-pJG4-5	MET12 gene was PCR amplified from genomic DNA using XhoI FW and XhoI RV primers and cloned in prey plasmid pJG4-5 after digesting both insert as well as vector backbone with XhoI. Clone confirmed by restriction digestion.
ABE 5500	STR3-pJG4-5	STR3 gene was PCR amplified from genomic DNA using XhoI FW and XhoI RV primers and cloned in prey plasmid pJG4-5 after digesting both insert as well as vector backbone with XhoI. Clone confirmed by restriction digestion.

2.3. OLIGONUCLEOTIDES

The list of various oligonucleotide primers used in this study is given in Table 3.

2.4. MEDIA

All the media, buffers and stock solutions were prepared using Millipore elix 5 deionized water unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch^2 (psi) pressures at 121ºC for 15 minutes, or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.22 µm (for heat labile compounds). Additional amino acid and nutrients were prepared as sterile stock and added as per requirements. Agar was added, if required, at a final concentration of 2.2%. Ampicillin was added at a final concentration of 100 µg/ml.

2.4.1. LB **(Luria–Bertani) Medium**	Yeast extract 5 g/l Tryptone 10 g/l NaCl 10 g/l pH of the above medium was adjusted to 7.0 with 1N NaOH
2.4.2. YPD **(Yeast extract Peptone Dextrose) Medium**	Yeast extract 10 g/l Peptone 20 g/l Dextrose 20 g/l
2.4.3 SD **(Synthetic Defined) Medium** **(per 1000 ml)**	YNB 1.7 g/l (Yeast Nitrogen Base) (without amino acids and ammonium sulphate) $(NH_4)_2 SO_4$ 5 g/l Dextrose 20 g/l Amino acids 80 mg/l (as required) pH was adjusted to 6.0–6.5.

Table 3: List of Oligonucleotides and their sequences used in this study

Oligo Name	**Sequence (5' to 3')**
STR3(600)-Xho1F	**GATCGACTCGAGTTTGAAGCCAGAACAG**
STR3-BamH1-R	**GATCGAGGATCCCATCTTTTTGCTTCTATGC**
STR3(441)-Xho1F	**GATCGACTCGAGAATTTGCCACACTGTTTTCC**
STR3(395)-Xho1F	**GATCGACTCGAGTCCGGCTGACTCATTC**
STR3(363)-Xho1F	**GATCGACTCGAGAGTGTGGCATGTGCTTC**
STR3(288)-Xho1F	**GATCGACTCGAGTGCTCCTACCTTGGAAG**
STR3(256)-Xho1F	**GATCGACTCGAGAAAGTGTGTAAAGGGGCC**
pRS426_F	**GCTGCAAGGCGATTAAGTTGGG**
STR3(600)-MET31/32_1st M MUT_FW	**GCCACACTGTTTTCCGTGGCGCCTTAGTCCGCGTTTGGTTATCCGGC**
STR3(600)-MET31/32_1st M MUT_RV	**GCCGGATAACCAAACGCGGACTAAGGCGCCACGGAAAACAGTGTGGC**

STR3(600)-MET31/32_2nd M MUT_FW	TCTGACTCTTTTTTGGAAAGTAAGGCATGTGCTTCACACAATATAATTCCC
STR3(600)-MET31/32_2nd M MUT_RV	GGGAATTATATTGTGTGAAGCACATGCCTTACTTTCCAAAAAAGAGTCAGA
STR3(600)-MET31/32_3rd M MUT_FW	GACTCATCGCATCTCAAAAGTAAGTAAAGGGGCCTTCTGGCC
STR3(600)-MET31/32_3rd M MUT_RV	GGCCAGAAGGCCCCTTTACTTACTTTTGAGATGCGATGAGTC

STR3(600)2-MET31/32_1st M MUT_FW	GCCACACTGTTTTCCGTGGCGTTTTTGTCCGCGTTTGGTTATCCGGC
STR3(600)2-MET31/32_1st M MUT_RV	GCCGGATAACCAAACGCGGACAAAAACGCCACGGAAAACAGTGTGGC
STR3(600)2-MET31/32_2nd M MUT_FW	TCTGACTCTTTTTTGGAAAGAAAAACATGTGCTTCACACAATATAATTCC
STR3(600)2 MET31/32_2nd M MUT_RV	TATATTGTGTGAAGCACATGTTTTTCTTTCCAAAAAAGAGTCAGAATGAG
STR3(600)2 MET31/32_3rd M MUT_FW	GGAAGACTCATCGCATCTCAAAAAAAAATAAAGGGGCCTTCTGGCCATATATCC
STR3(600)2-MET31/32_3rd M MUT_RV	GGATATATGGCCAGAAGGCCCCTTTATTTTTTTTGAGATGCGATGAGTCTTCC
ScSTR3BamHIFW	GTCAAGGATCCATGCCGATCAAGAGATTAGATAC
ScSTR3Xho1REV	TCACGCTCGAGTTACAATTTCGAACTCTTAATATTC
MET12-BamHI-F	GATCAGGGATCCATGTCCATCAGAGATTTATATCATGCG
MET12-Xho1-R	GATCAGCTCGAGTTAGGCTTGGTCGAGTAACATTTCCC
ScSTR3XhoIFW	GTCAACTCGAGATGCCGATCAAGAGATTAGATAC
MET12-XhoI-F	GATCAGCTCGAGATGTCCATCAGAGATTTATATCATGCG

2.5 BUFFERS AND STOCK SOLUTIONS

2.5.1. Ampicillin Stock Solution (100 mg/ml)

The required amount of ampicillin (sodium salt) was dissolved in the required volume of deionized water, and it was filter-sterilized using 0.2 μm filter membrane. It was stored at -20°C in aliquots in micro centrifuge tubes.

2.5.2. GSH Stock Solution (200 mM)

The required amount of glutathione (reduced form) was dissolved in 1 ml of deionized water and was filter-sterilized using 0.2 μm filter membrane. It was used for further dilutions and was stored at -20°C.

2.5.3. Methionine Stock Solution (200 mM)

The required amount of methionine was dissolved in 1 ml of deionized water and was filter-sterilized using 0.2 μm filter membrane. It was used for further dilutions and stored at 4°C.

2.5.4. Cysteine Stock Solution (200 mM)

Fresh stock of cysteine was prepared by dissolving required amount of cysteine in1 ml of deionized water and was filter sterilized using 0.2 μm filter membrane.

2.5.5. 50% Glycerol (used for preparing –80°C stocks of *E. coli*)

2.5.6. Alkaline Lysis Buffers (Plasmid DNA preparation from *E. coli*)

a) Solution-I (Resuspension Solution)	50 mM Glucose 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
b) Solution-II (Lysis Solution) (freshly prepared)	0.2 N NaOH (freshly diluted from a 10 N stock) 1% SDS (freshly diluted from a 10% stock) Stored at room temperature.
c) Solution-III (Neutralization Solution)	5 M Potassium acetate 60 ml Glacial acetic acid 11.5 ml Deionized water 28.5 ml The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. It was stored at 4ºC.
d) TE Buffer (Tris-EDTA) (pH 8.0)	10 mM Tris-HCl (pH 8.0). 1 mM EDTA (pH 8.0).
e) TE-RNAse (stock prepared at 10 mg/ml)	Working stock 20 µg/ml in TE Buffer, pH 8.0.
f) PCI (Phenol-chloroform-isoamyl alcohol) Solution (100ml)	Phenol 50 ml [Equilibrated with Tris-HCl (pH 7.6)] Chloroform 48 ml Isoamyl alcohol 2 ml Stored at 4°C in dark brown bottle.

2.5.7. Agarose Gel Electrophoresis Reagents

a) 1× TAE (Tris-acetate-EDTA) Buffer (per 1000 ml) (prepared from 50× TAE stock)	40 mM Tris-acetate. 1mM EDTA (pH 8.0). Autoclaved and stored at room temperature.
b) Orange-G dye (Gel loading dye, 6X)	0.25% orange-G and 30% glycerol
c) 0.7-1% Agarose gel in 1× TAE	
d) Ethidium Bromide (10 mg/ml) Stock	Final working concentration used at 0.5 µg/ml.

2.5.8. Solutions for preparation of chemical competent *E. coli* cells [19]

a) SOB	Bactotryptone 20 g Bacto yeast extract 5 g NaCl 0.5 g Above mentioned components were dissolved in 950 ml of water. 10 ml of 250 mM KCl was added and pH was adjusted to 7 with 5N NaOH, volume was made up to 995 ml and autoclaved. Just before use, 5 ml of filter sterilized 2 M $MgCl_2$ was added.
b) SOC	SOB + 20 mM Glucose
c) 10% glycerol	

2.5.9. Yeast Transformation Solutions (*S. cerevisiae*) [20]

a) 0.1 M Lithium acetate in TE (pH 7.5)

b) 50% PEG-3350 in 0.1 M Lithium acetate in TE (pH 7.5).

SECTION B: METHODS

2.6 Growth and maintenance of bacteria and yeast strains

The *Escherichia coli* strains DH5α was routinely grown at LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The *S. cerevisiae* strains were regularly maintained on YPD medium and grown at 28-30°C. The yeast transformants were selected and maintained on SD medium with supplement as per requirements.

2.7 Recombinant DNA methodology (restriction digestion, ligation, transformation of *E. coli,* PCR amplification etc.

All the molecular techniques used in the study for manipulation of DNA, protein, bacteria and yeast were according to standard protocols (Kaiser et al., 2000; Sambrook et al., 1989) or as per manufacturers' protocol, unless specifically mentioned.

2.8 Growth of cells for induction

The STR3-promoter-GFP fusion construct was transformed in different strain backgrounds. Strains were grown overnight in complete media without uracil and reinoculated in fresh medium and grown in presence of different sulphur and nitrogen sources for 5 hours.

2.9 GFP Assay

After growing cells in different induction conditions, cells were harvested at 4°C, followed by washing with cold water and resuspended in water to an OD_{600} of 1.0 and GFP fluorescence was measured in a spectrophotometer at Excitation:488nm and Emission: 510nm.

2.10 Transformation of yeast

The transformation of *S. cerevisiae* strains was carried out by lithium acetate method. *S. cerevisiae* cultures were grown in YPD at 30°C with shaking for 16-24 hrs and then reinoculated in fresh YPD to an initial OD_{600} of 0.1, cells were allowed to grow at 30°C for 4-5 hrs with shaking. Cells were harvested at 6000 rpm for 5 min, then were washed with sterile water followed by subsequent wash with 0.1 M lithium acetate solution (prepared in

TE, pH 7.5) and were finally resuspended in the same solution. Cells were incubated at 30ºC for 30 min with shaking. The cells were spun down, suspended in 0.1 M lithium acetate solution to a cell density of 1×10^9 cells/ml and divided into 100µl aliquots. Approximately 50 µg (5 µl of 10 mg/ml stock solution) of heat denatured, salmon sperm carrier DNA, followed by 0.3 µg- 0.7µg of plasmid/DNA fragment were added to each aliquot and whole cell suspension was incubated at 30ºC for 30 min. After the incubation, 0.3 ml of 50% PEG 3350 (prepared in 0.1 M lithium acetate, pH 7.5) was added to each tube, mixed well and again kept at 30ºC for 45 min. The cell suspensions were subjected to heat shock at 42ºC for 10 min. and the cells were allowed to cool to room temperature. The cells were pelleted down at 7000 rpm for 3 min. The cell pellet was resuspended in sterile water and appropriate volume of cell suspension was plated on selection plates.

2.11 Construction of Site-directed mutants of STR3 promoter

The mutations in *STR3* promoter were introduced by site directed mutagenesis using the splice overlap extension strategy. The different mutagenic oligonucleotides pairs used for generation of these mutants are given in Table 2.

2.12 Yeast Two Hybrid assay

Yeast two hybrid studies were carried out using the DupLexA yeast two hybrid system of Origene technologies Inc., MD (USA). This system was initially described by R.Brent and co-workers (Gyuris *et al.*, 1993). pEG202 was used as the bait plasmid and *MET12* was expressed as a fusion protein with LexA DNA binding domain. pJG4-5 plasmid was used as the prey plasmid and *STR3* was expressed as a fusion protein with Gal4 activation domain. Interactions between the two proteins result in the formation of a transcriptional competent complex having both LexA DNA binding domain and Gal4 transcription activation domain. The interactions were checked using two reporters. pSH18-34 plasmid having 6LexA-operator upstream of β-galactosidase was used for Blue-White selection on X-Gal plates. Interaction among the two proteins results in the expression of β-galactosidase and blue colour on X-Gal plates. A second reporter was provided by the yeast strain (EGY48) used in the system. EGY48 has 6LexA-operator upstream of *LEU2* gene. Interaction among the two proteins results in the expression of *LEU2* and allows the growth on CM-Leu plates of the leucine auxotroph EGY48.

pSH17-4 was used as positive control. In this plasmid ADH1 promoter expresses a LexA-Gal4 fusion protein. pRFHM1 was used as a negative control in the study, its expresses LexA fused to homodomain of bicoid protein that does not function as an activation domain in yeast.

The three plasmids (Reporter, Bait pEG202-MET12 and Prey pJG4-5 STR3) were transformed into EGY48 strain and transformants were selected on SD-Ura-His-Trp+Leu plates. True transformants were patched on CM+Leu, CM-Leu and CM-X-Gal plates. Positive interactions show blue colour on CM+X-Gal plates and growth on CM-Leu plates. Bait constructs alone were also checked for the autoactivation. Repeated the same by using *STR3* in bait and *MET12* in prey.

Chapter 3

Results and Discussion

Results and Discussion

3.1 Phylogenetic footprinting of *STR3* promoter reveals several conserved motifs

Phylogenetic footprinting is a technique to identify conserved regions within non-coding regions of DNA by multiple sequence alignment of the orthologous sequences of closely related species (Kohli et al., 2004). This technique can be used to identify motifs with physiological significance.

Chromosomal Locus of STR3: Chromosome VII

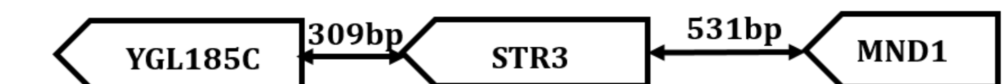


Figure 3.1: Schematic representation of chromosomal locus of *STR3* showing intergenic distance of 531bp between *STR3* and its upstream gene *MND1*

To obtain insights on *STR3* regulation, we aligned ~600kb region of the *STR3* promoter of following *Saccharomyces* species - *S. cerevisiae, S. mikatae, S. paradoxus, S. uvarum, S. bayanus* and *S. kudriavzevii.*

The multiple sequence alignment is shown in Figure 3.2.

MND1

```
Sacc.cere  422  AAAAGAATTTGGAATTCCAGAAGAGTTCAAGGAATTTACGGAGGTTTAAC  471
Sacc.mika  406  AAAAGAATTTGGAATTCCAGAAGAGTTCAAAGAGTTCACAGATATTTAGC  455
Sacc.para  451  AAAAGAATTTGGAATTCCAGAAGAGTTCAAAGAGTTTACAGAGGTTTAAC  500
Sacc.uvar  415  AAAGGAGTTCGAAATCCCGGAAGAGTTCAAGGAGTTCGCGGATGTATAGG  464
Sacc.baya  415  AAAGGAGTTCGAAATCCCGGAAGAGTTCAAGGAGTTCGCGGATGTATAGG  464
Sacc.kudr  432  AAAAGAATTCGGAATTCCAGAAGAATTCGAAGAGCTTACTGACATCTAGT  481

Sacc.cere  472  CTTCATGCAC------TATACACGAA-AGAACAAAGTGTCTTGATTATT  514
Sacc.mika  456  C------CAC------TATATATCTA-A-AACCAAAGAATTCTGATTATC  491
Sacc.para  501  TCTCATTCACAA----TACACACATA-GGAACAAAACTAACCTAATTATC  545
Sacc.uvar  465  GCACACACACAAATATTATACATATATATATATAAATAGGCATATGTACC  514
Sacc.baya  465  GCACACACACAAATATTATACATATATATATATAAATAGGCATATGTACC  514
Sacc.kudr  482  C------CACAA----CGTACACACG-ATATGAATACAAATAGACACATA  520

Sacc.cere  515  -ACATCTACAT---TTCCAGG-----CATAGAGC----------TTGCA-  544
Sacc.mika  492  -ACATATATGTACTTTGCAAAACACACATGGAAAAGAGAACCATTTCCAA  540
Sacc.para  546  -GCATATATAC---TTGCAAA-----CATATACA----------CTCCA-  575
Sacc.uvar  515  CTCGCAAACGT---ATACAAA-----TATTAGCCGCTGTGTCGCTCTGTC  556
Sacc.baya  515  CTCGCAAACGT---ATACAAA-----TATTAGCCGCTGTGTCGCTCTGTC  556
Sacc.kudr  521  -TTTTACATCC---TTGCTAG---CACTCAGAGA----------TGAAAA  553
```

```
Sacc.cere  545  --GATTAGCCGCCCATAAATTTGCCACACTGTTTTC-CG---TG-GCGCC  587
Sacc.mika  541  AAGATTAGCCGCCCATAGCTTTACTACACAGTTACAGCAATGCG-GCGCC  589
Sacc.para  576  --GATTAGCCGCCCATAAATTCACCAC--TGTTTTT-CGATTTG-GCGCC  619
Sacc.uvar  557  TAGATTAGCCGCCCA-CGCTACGTTACACAGTTTTTTTGCTGTGGCGCC  605
Sacc.baya  557  TAGATTAGCCGCCCA-CGCTACGTTACACAGTTTTTTTGCTGTGGCGCC  605
Sacc.kudr  554  ACGATTAGCCGCCC--AAACTCGCCACACATTTTTCGCAACGTAAGCGCC  601

Sacc.cere  588  ACAGTCCGCGTTTG-GTT-ATCCGGCTGACTCATTCTGACTCTTTTTTGG  635
Sacc.mika  590  ACAGTCCGTCCGAG-CCCCCTCCAGCTGACTCATTCTGACTCTTTTCTTG  638
Sacc.para  620  ACAGTCCGCGAGCG-GTCCATCCGGCTGACTCATTCTGACTCTTTCCTGG  668
Sacc.uvar  606  ACAGTTTTGCCGACCGCACATCCGGCTGACTCATTTCGACCGCTTTTCCT  655
Sacc.baya  606  ACAGTTTTGCCGACCGCACATCCGGCTGACTCATTTCGACCGCTTTTCCT  655
Sacc.kudr  602  ACAGTTTATCAGTGCACTTATCCGGCTGACTCATTTTGACTCTTTTTCAA  651

Sacc.cer   636  AAAGTGTGGCATGTGCTTCACACAATATAATTCCCGTGAGCACAGCATG-  684
Sacc.mika  639  GAC-TGTGGCATATGTCCCGCACAATATACTTTCTATCAGCAC---ACG-  683
Sacc.para  669  AAC-TGTGGCATGTGTTCCACACAATACGATTTCTGTGAGCACAGCATG-  716
Sacc.uvar  656  GCGTTGTGGAACGCACTCC-CGCA------TTCACAGTTGTGCCTCTAGC  698
Sacc.baya  656  GCGTTGTGGAACGCACTCC-CGCA------TTCACAGTTGTGCCTCTAGC  698
Sacc.kudr  652  CAC-TGTGGCATGTTTTCCGCGCAACATGCTTCTTACCATCACAGCGAGT  700

Sacc.cere  685  CTT-------CGGCCGA-AGTCTGTTGGAGGCATATGCTC----CTACC  722
Sacc.mika  684  CTTTCCGTTTCGGCCGAGACTGGACTGGGGAATATGGTC----CTACC  729
Sacc.para  717  CTTTCCGCTTCGGCCGAGAATCTATTGGAGGCATATGCTC----CTACC  762
Sacc.uvar  699  CT--------CGGATTG---CCCTAGTAGAGGCAACTGGTTGCTGCTACT  737
Sacc.baya  699  CT--------CGGATTG---CCCTAGTAGAGGCAACTGGTTGCTGCTACT  737
Sacc.kudr  701  CTTCCGGTCTCGGCCGAGACTCTGTGGGAAGCATATGTTC----CTACC  746

Sacc.cere  723  TTG--GAAGACTCATCGCATCTCAAAAGTGTGTAAAGG---GGCCTTCTG  767
Sacc.mika  730  CTGAAGAAGACTCATCGCATCGCAAAAGTGTGTAAATGATGGGTACTATG  779
Sacc.para  763  TTG--GAAGACTCATCGCATCGCAAAAGTGTGGAAAGG---GGCCTTCTG  807
Sacc.uvar  738  CTGC-TAGGACACATCGCATCGCAAAAGTGTGTAAACGT--GTAAGCGTG  784
Sacc.baya  738  CTGC-TAGGACACATCGCATCGCAAAAGTGTGTAAACGT--GTAAGCGTG  784
Sacc.kudr  747  CTGG-GGAGATTCATCGCA-----AAAGTGTGTAAACG--GGGCCCTCTG  788
```

```
Sacc.cere   768  GCCATA--------TATCCTCTGAGACCTGGTTGAA-----ATGTGGCCA   804
Sacc.mika   780  CCCTTAACCTCCTGTATCGTCCCAGACCAGATCGGATAGGAATGAGACCA   829
Sacc.para   808  TCCATC--------TATCCTCCAGGACCTGACTGAA-----ATGTGGCCA   844
Sacc.uvar   785  GAAGTCCCT-----TAGCCTTCTTATCCGCTCCGCA--------ACATCG   821
Sacc.baya   785  GAAGTCCCT-----TAGCCTTCTTATCCGCTCCGCA--------ACATCG   821
Sacc.kudr   789  CACACAACCTTTCGTACCCTCTATGACTCGGTTGAA------TGTGATCA   832

Sacc.cere   805  GAAGCAAAGCTGATAAAAACAACTGGCGCATACGTAGCTATATTTTAAAT   854
Sacc.mika   830  GAAGCAAAGTTAACACACGCAACGTGC----------------------T   857
Sacc.para   845  GAAGCAGTGTTAACAAAAGTAAAGGC------------------------   871
Sacc.uvar   822  GGAG--GGGTTACGATAGGCAACTCGT-----------------------   846
Sacc.baya   822  GGAG--GGGTTACGATAGGCAACTCGT-----------------------   846
Sacc.kudr   833  AAAGAAGAAACGCAACGAAGGAAGTAGT----------------------   860

Sacc.cere   855  AAATAAATAAATAAATATATATATATATATATATATATATATACATATAT   904
Sacc.mika   858  ACGCACATAGTTGCGCACACACAGCCAAGAATA--TATATATATATATAT   905
Sacc.para   872  -------------AACACTTGTAGCTTTATATA-ATATATATATACATAT   907
Sacc.uvar   847  --------------AAATACGAATGCATACGTACATGTATATATATATAT   882
Sacc.baya   847  --------------AAATACGAATGCATACGTACATGTATATATATATAT   882
Sacc.kudr   861  ---------GGGCAATAAACATAGTTGCAAATGTAGATACAAATATATAT   901

Sacc.cere   905  ATACAGTCATTGCCACACATCCCATAGCTCTGTGTGCTTACAGTTCATTA   954
Sacc.mika   906  ACTAGATTTTAGCTCTACATTGTACTGG-ATCTGTGCTTGACGTTCAGTA   954
Sacc.para   908  ATATAGCCATAGCTATACATTTTACAGA-CTCTGTACTTACAGTTCATTA   956
Sacc.uvar   883  AGCGGGTTATACGTATACATTTTGCAGG-TTCTGTTCTTTAAGCTTATCG   931
Sacc.baya   883  AGCGGGTTATACGTATACATTTTGCAGG-TTCTGTTCTTGAAGCTTATCG   931
Sacc.kudr   902  AGTGGGACATAGCTATACTTTTAACTTG-CACAGCGCCTGAAGTTTATCG   950
                                                                -1
Sacc.cere   955  A--AAACAAAAAATATCA-AGCAAAC-AACAAAAGCATAGAAGCAAAAAG   1000
Sacc.mika   955  A--AAATAAACAGAACCC-AACAAAT-AACAAACTAACAGAAAGAAGAAG   1000
Sacc.para   957  A--GAGTAAAAAAT--TA-AGCAAAC-AAAAAGCCAAAACAAGCAAAAAAA   1000
Sacc.uvar   932  GTTAAACTGCAAATA-TATAACAAACAAGCAAACAAGCAAACAAGCAAAC   980
Sacc.baya   932  GTTAAACTGGAAATA-TATAACAAACAAGCAAACAAGCAAACAAGCAAAC   980
Sacc.kudr   951  A--AAATAAAAAAGAATACAACAGACCAACAGACTAACACAACTTGAAAA   998

Sacc.cere        --------------------
Sacc.mika        --------------------
Sacc.para        --------------------
Sacc.uvar   981  ATACTAACAATCTAATCAAC   1000
Sacc.baya   981  ATACTAACAAGCGAATCAAC   1000
Sacc.kud    999  AT------------------   1000
```

Figure 3.2: Phylogenetic footprinting of ~600bp region of *STR3* promoter of different *Saccharomyces* species

The intergenic sequence was 531bp between *STR3* and upstream gene *MND1*(Figure 3.1).We therefore focused on that region of the promoter.(Figure3.3)

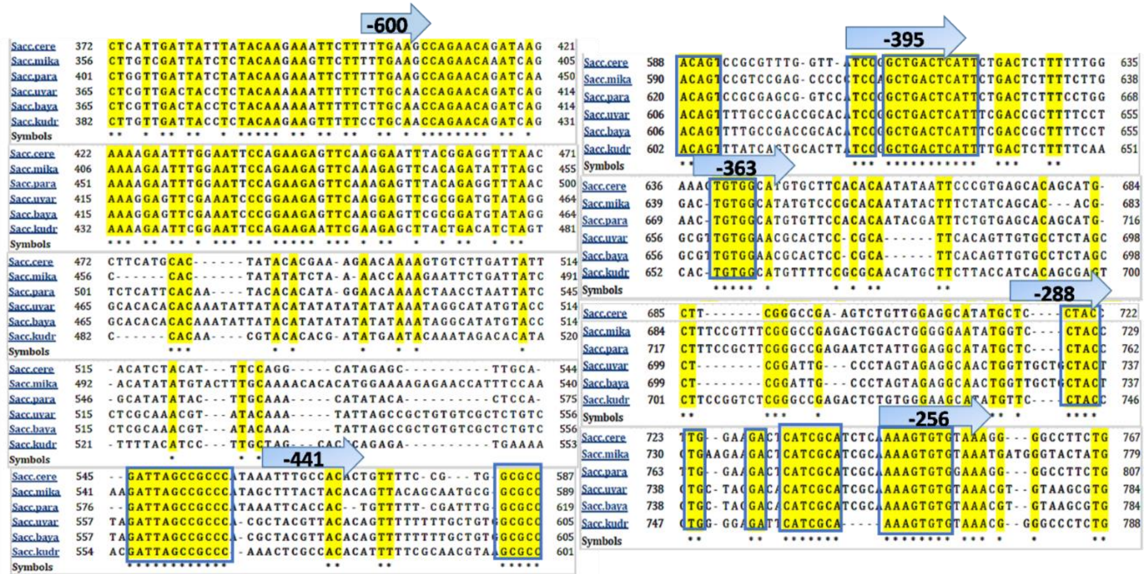


Figure 3.3: Phylogenetic footprinting of *STR3* promoters of different *Saccharomyces* species. The yellow regions in blue boxes highlight conserved regions in *STR3* promoter.

The phylogenetic footprinting of *STR3* promoter revealed many conserved motifs. Using YEASTRACT database (Teixeira et al., 2014) we predicted transcription factors which can probably bind to these conserved sequences. (Figure 3.5)

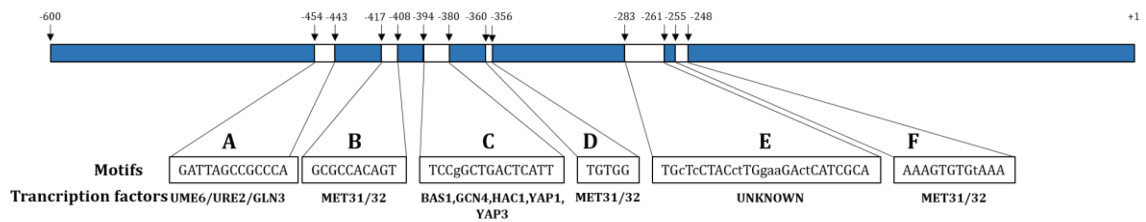


Figure 3.4 : Schematic representation of conserved motifs present in *STR3* promoter and transcription factors predicted by YEASTRACT which may probably bind to these motifs.

		Observed motif from alignment	Transcription factor consensus motif
	A	GATTAGCCGCCCA	AGCCGCCNA (Ume6)
	A	GATTAGCCGCCCA	GAT[TA]AG (Gln3)
	B	GCGCCACAGT CGCGGTGTCA	AAACTGTGGC (Met31,Met32)
	C	TCCgGCTGACTCATT	TGACTC (Bas1,Gcn4)
	C	TCCgGCTGACTCATT	CCAGC (Hac1)
	C	TCCgGCTGACTCATT	TGACTCA (Yap1,Yap3)
	D	TGTGG	AAACTGTGGC (Met31,Met32)
	E	TGtTcCTACctTGgaaGActCATCGCA	*
	F	AAAGTGTGtAAA	AAACTGTGGC (Met31,Met32)

Figure3.5 : Showing all the putative transcription factors which can bind to conserved sequences present in *STR3* promoter along with observed and consensus sequences of transcription factors. The underlined regions correspond to transcription factor binding sites.

Brief description about putative transcription factors :-

Ume6- It is a histone deacetylase complex unit. It is a key regulator of nitrogen repression and meiotic development. (Strich et al., 1994).

Gln3- Gln3 is a transcriptional activator of genes which show nitrogen catabolite repression. Its localization and activity is regulated by Ure2p and quality of nitrogen sources (Daignan-Fornier and Fink, 1992).

Ure2- Transcriptional regulator of nitrogen catabolite repression and inhibits Gln3 transcription in good nitrogen sources. (Daignan-Fornier and Fink, 1992).

Met31/Met32- These have been already described in the introduction.

Bas1:- It regulates basal and induced expression of genes of purine and histidine biosynthesis pathways. (Courchesne and Magasanik, 1988; Magasanik and Kaiser, 2002).

Gcn4:- bZIP transcriptional activator of amino acid biosynthetic genes. It responds to amino acid starvation and its expression is tightly regulated at both the transcriptional and translational levels. (Hinnebusch and Natarajan, 2002).

Hac1:- bZIP transcription factor which regulates the unfolded protein response via UPRE binding and membrane biogenesis. ER stress-induced splicing pathway and DNA replication stress facilitates efficient Hac1p synthesis (Mori et al., 1996).

Yap1:- bZIP transcription factor which is required for oxidative stress tolerance. It also mediates resistance to cadmium and relative distribution to the nucleus increases upon DNA replication stress (Kuge et al., 1997; Okazaki et al., 2007).

Yap3:- bZIP transcription factor and it is like activator protein 1(AP-1) which is a transcription regulator that regulates gene expression in response to a variety of stimuli including cytokines, growth factors, stress, bacterial and viral infections (Fernandes et al., 1997).

3.2 Creation and validation of STR3-pro-GFP fusion construct

To investigate the regulation of *STR3* more systematically we created a STR3-GFP reporter in-frame fusion.(Figure3.6) Before proceeding toward the expression analysis of *STR3* under our experimental conditions, we first validated the STR3-pro-GFP construct, since *STR3* is known to be regulated by sulphur sources. We examined the expression of *STR3* under sulphur limiting conditions and compared the results with *MET15* whose upregulation in sulphur limiting conditions is also well established. The STR3-pro-GFP plasmids were transformed into the wild type yeast strain BY4742 (ABC734). The effect of different sulphur sources on *STR3* and *MET15* gene expression were examined by growing cells bearing either STR3-pro-GFP plasmids or MET15-pro-GFP plasmids in different sulphur sources like methionine, cysteine and glutathione (see Methods).

De-repression of both *STR3* and *MET15* was observed in sulphur limiting conditions confirming that STR3-pro-GFP construct was functional. The expression pattern of *STR3* reflects strong transcriptional repression by methionine and cysteine sulphur sources at

500µM. On the contrary, glutathione did not show any significant repression as it is known to be a non-repressing sulphur source.(Figure 3.7)

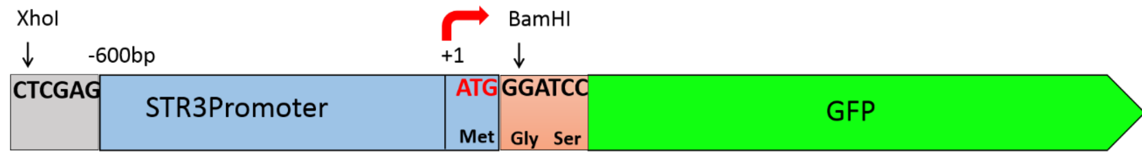


Figure 3.6: Schematic representation of in-frame fusion product of STR3-promoter-GFP reporter construct

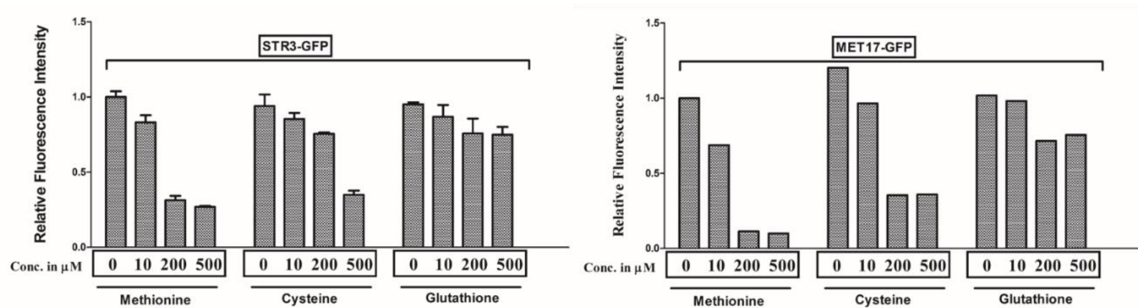
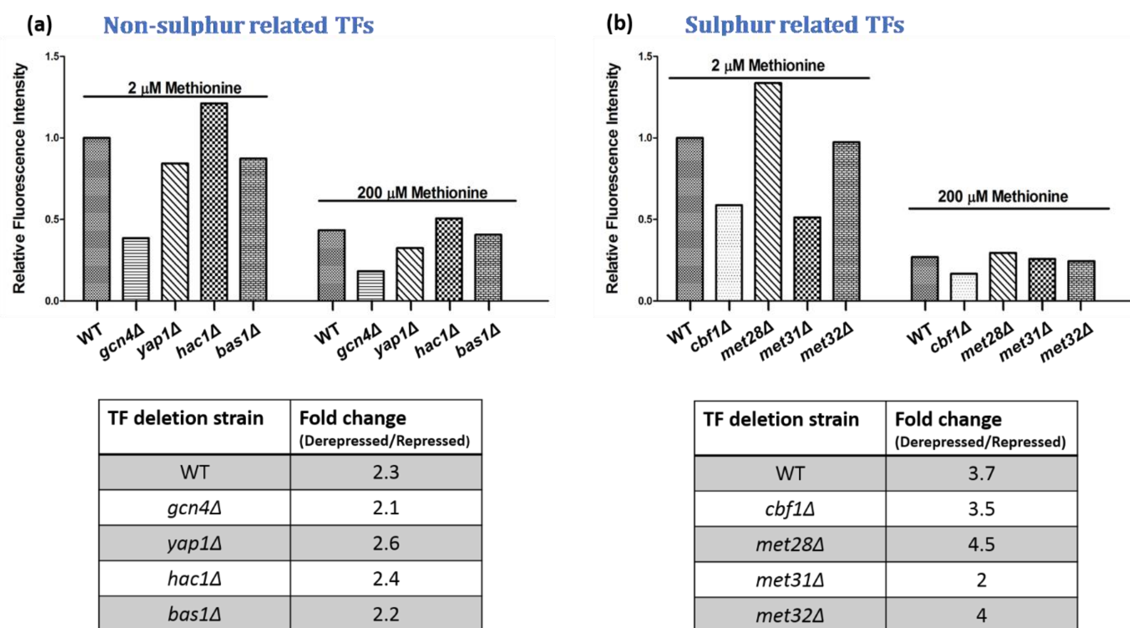


Figure 3.7: Effect of methionine, cysteine and glutathione on *STR3* and *MET15* expression.

3.3 Investigating the regulation of *STR3* in different transcription factor deletion backgrounds

To determine if the predicted transcription factors might have a role in *STR3* regulation, we examined the regulation of *STR3* in different transcription factor deletion genetic backgrounds under sulphur de-repressed and repressed conditions. (Figure 3.8)



TF deletion strain	Fold change (Derepressed/Repressed)
WT	2.3
gcn4Δ	2.1
yap1Δ	2.6
hac1Δ	2.4
bas1Δ	2.2

TF deletion strain	Fold change (Derepressed/Repressed)
WT	3.7
cbf1Δ	3.5
met28Δ	4.5
met31Δ	2
met32Δ	4

Figure 3.8: *STR3* regulation in strains deleted for different transcription factors (a) Non-sulphur related transcription factors (b) Sulphur related transcription factors.

We observed that among non-sulphur related transcription factors, *gcn4*Δ leads to drastic decrease in *STR3* expression under de-repressed condition (2µM Methionine) which suggests an important role of GCN4 for complete de-repression of *STR3*.

In case of the sulphur related transcription factors, decrease in de-repression values of *STR3* in *cbf1*Δ and *met31*Δ strains suggesting the role of *CBF1* and *MET31* in transcriptional regulation of *STR3*. Furthermore, the decline in extent of repression of *STR3* gene under sulphur sufficient conditions in a *met31*Δ strain is indicative of a greater role of *MET31* in regulation of *STR3*.

3.4 Deletion analysis of *STR3* promoter in sulphur de-repressed and repressed conditions

To identify the cis-regulatory elements involved in the regulation of *STR3*, we carried out deletion analysis of *STR3* promoter. GFP based assays were performed with yeast strains transformed with truncated promoter-fusion constructs (-600, -440, -395, -363, -288, -256) (Figure 3.9)

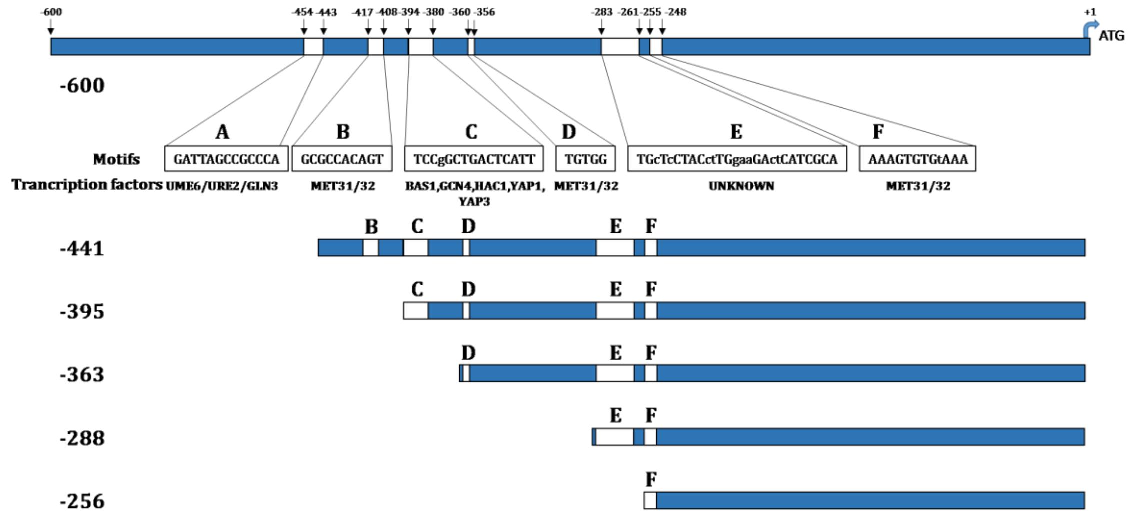


Figure 3.9: Schematic representation of constructs made for deletion analysis of *STR3* promoter

We observed that MOTIF A and B did not affect the expression of *STR3* under either sulphur de-repressed or repressed conditions. This suggests that MOTIF A and B are not crucial for expression of *STR3*. However in the -363 promoter construct, deletion of MOTIF C (putative GCN4 motif) leads to sufficient decrease in expression in sulphur de-repressed conditions. This is consistent with our previous result that GCN4 indeed is crucial for full de-repression of *STR3*.(Figure 3.10)

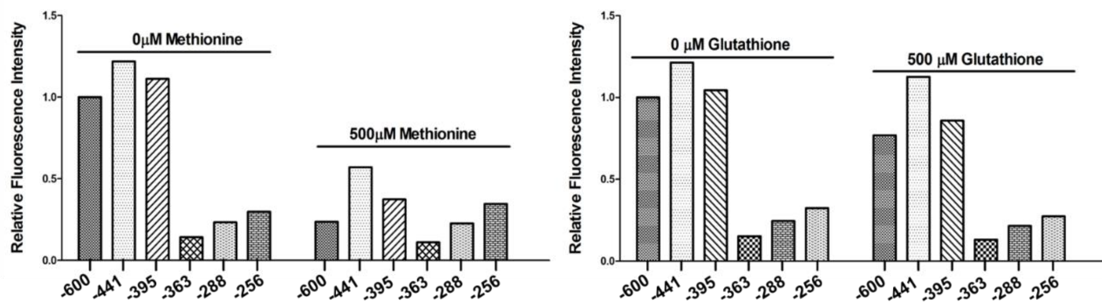


Figure3.10: Deletion analysis of *STR3* promoter by methionine and glutathione sulphur sources

3.5 Mutational analysis of MET31/32 motifs for their involvement in sulphur regulation

We carried out 2 bp and 5 bp mutation in putative MET31/32 Motif B,D and F. 2bp mutation in each of the motif leads to decrease in extent of de-repression value of *STR3* but did not affect the repressed expression significantly.(Figure 3.11)However this was not consistent with the 5bp mutation results. These experiments need to be repeated. From these results we concluded that may be these MET31/32 motifs are working redundantly.

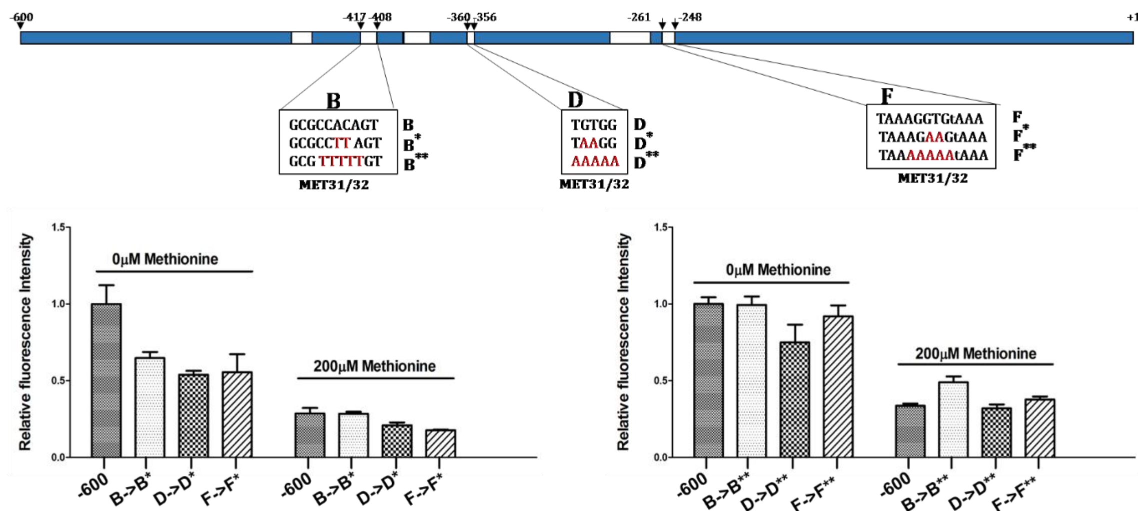


Figure 3.11: Identification of functionally important MET31/32 motifs in *STR3* promoter through mutational analysis

3.6 *STR3* regulation during intracellular cystine overload

Previous experiments in the lab have examined how cells responded to cysteine or cystine overload. In these experiments, either the cysteine transporter (YCT1) or a cystine transporter (CgCYN1) were overexpressed and these cells were treated with 500µM cysteine and cystine respectively. Microarray done under these conditions revealed that *STR3* was upregulated (Deshpande et al., 2017).We therefore thought of confirming these results with the STR3-pro-GFP reporter constructs in cells expressing the cystine transporters under similar conditions.

In contrast to the microarray results, we observed a downregulation of *STR3* (Figure3.12) with the 600bp-promoter GFP construct. The discrepancy of this data with microarray data is not clear and needs to be further investigated. The experiments were also done in a *yct1*Δ background to minimize any effects from residual cysteine.

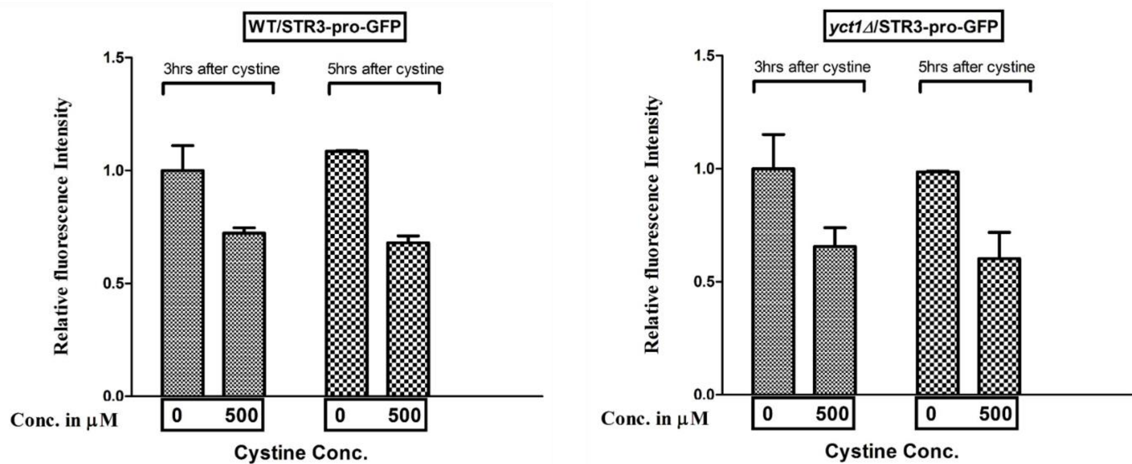


Figure 3.12: Effects of elevated levels of extracellular cystine on *STR3* regulation in wild type and *yct1*Δ *strain*

3.7 Role of conserved MOTIF A (Ume6 and Gln3 binding motif) in nitrogen regulation of *STR3*

Motif A corresponds to site for Ume6 and Gln3 binding motif. These are transcription factors involved in nitrogen regulation. To examine regulation of *STR3* by nitrogen sources, the reporter assay was carried out by growing cells in repressing (ammonium sulphate, ammonium chloride) and in non-repressing nitrogen sources (sodium glutamate). The nitrogen assimilatory pathway is known to be de-repressed by glutamate. No significant difference in de-repression value of cells grown in repressible and non-repressible nitrogen sources was observed and therefore it appears that *STR3* is not under nitrogen regulation.(Figure3.13) To further investigate the nitrogen regulation of *STR3* we examined STR3-promoter-GFP reporter in strains having *ume6Δ* and *ure2Δ* which are repressors of nitrogen regulatory pathway. However, we did not observe any increased activity. We also checked in *gln3Δ* background. GLN3 is a transcriptional activator of nitrogen regulatory network. But, no change in activity was observed. (Figure3.13)

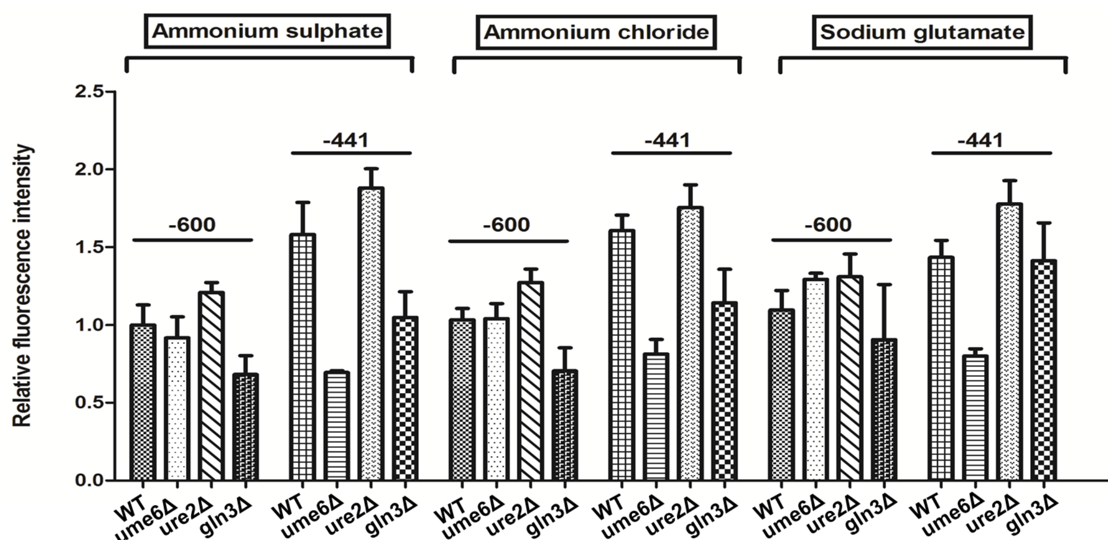


Figure 3.13: Effect of different nitrogen sources and regulators of nitrogen pathway on *STR3* regulation

3.8 STR3 expression analysis in *met12Δ and met12Δmet15Δ*

Previous experiments in our lab have shown that strains bearing a *met12*Δ*met15*Δ double deletion are unable to utilize GSH/cysteine as sulphur source. In contrast *met15*Δ strains which are organic sulphur auxotrophs were able to grow on GSH/cysteine. This block in utilization of GSH/cysteine by *met12*Δ*met15*Δ double deletion strain was eventually traced to the *STR3* step.(Bhatia M. unpublished) These studies have suggested that *met12*Δ was interfering with *STR3* activity. To evaluate whether the block is at the transcriptional level we investigated whether the expression levels of *STR3* differ in WT and *met12*Δ*met15*Δ. We also examined *STR3* expression levels in *met13*Δ and *met13*Δ*met15*Δ strain as *MET12* is a sequence homolog of *MET13* (though functionally they are different).

We observed comparable de-repression of *STR3* in both WT and *met12*Δ*met15*Δ suggesting that there is no transcriptional block at the *STR3* step in *met12*Δ*met15*Δ. (Figure3.14)

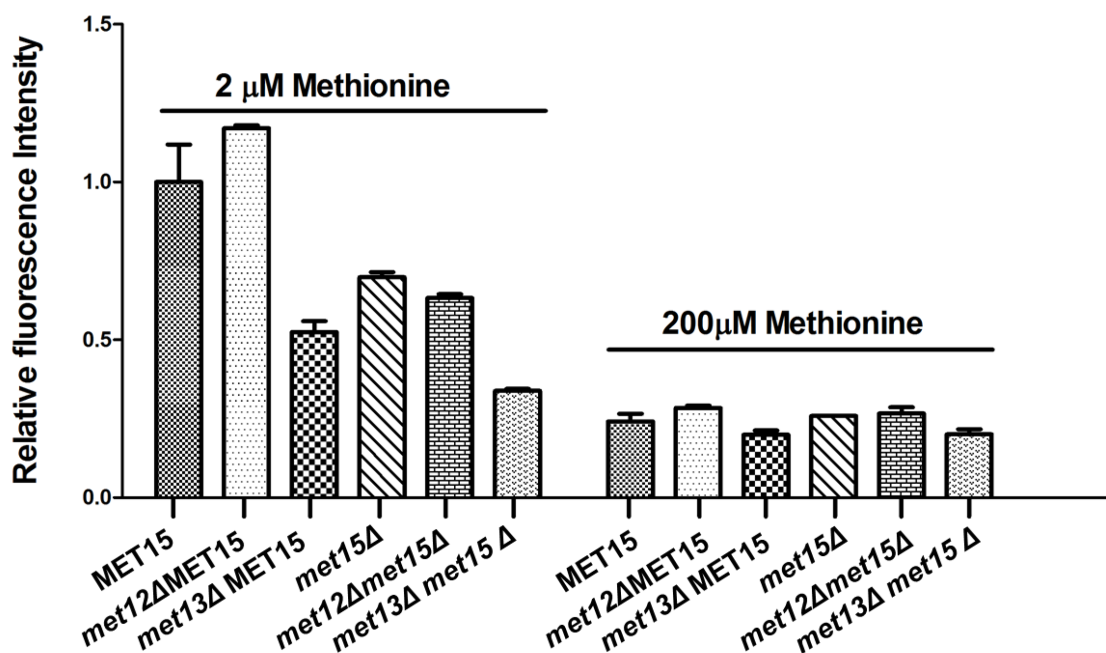


Figure 3.14: Checking transcriptional block at *STR3* step in *met12*Δ*met15*Δ

3.9 Yeast Two Hybrid Assay to check physical interaction between *MET12* and *STR3*

Since no transcriptional block of *STR3* by *met12*Δ*met15*Δ was observed, we examined whether physical interaction between *MET12* and *STR3* might explain the phenotype observations with *met12*Δ*met15*Δ.This was done by using yeast two hybrid assay. In one case we used *MET12* as bait and *STR3* as prey and in other case we used *STR3* as bait and *MET12* as prey. However in both the cases we could not observe any physical interaction. (Figure 3.15)

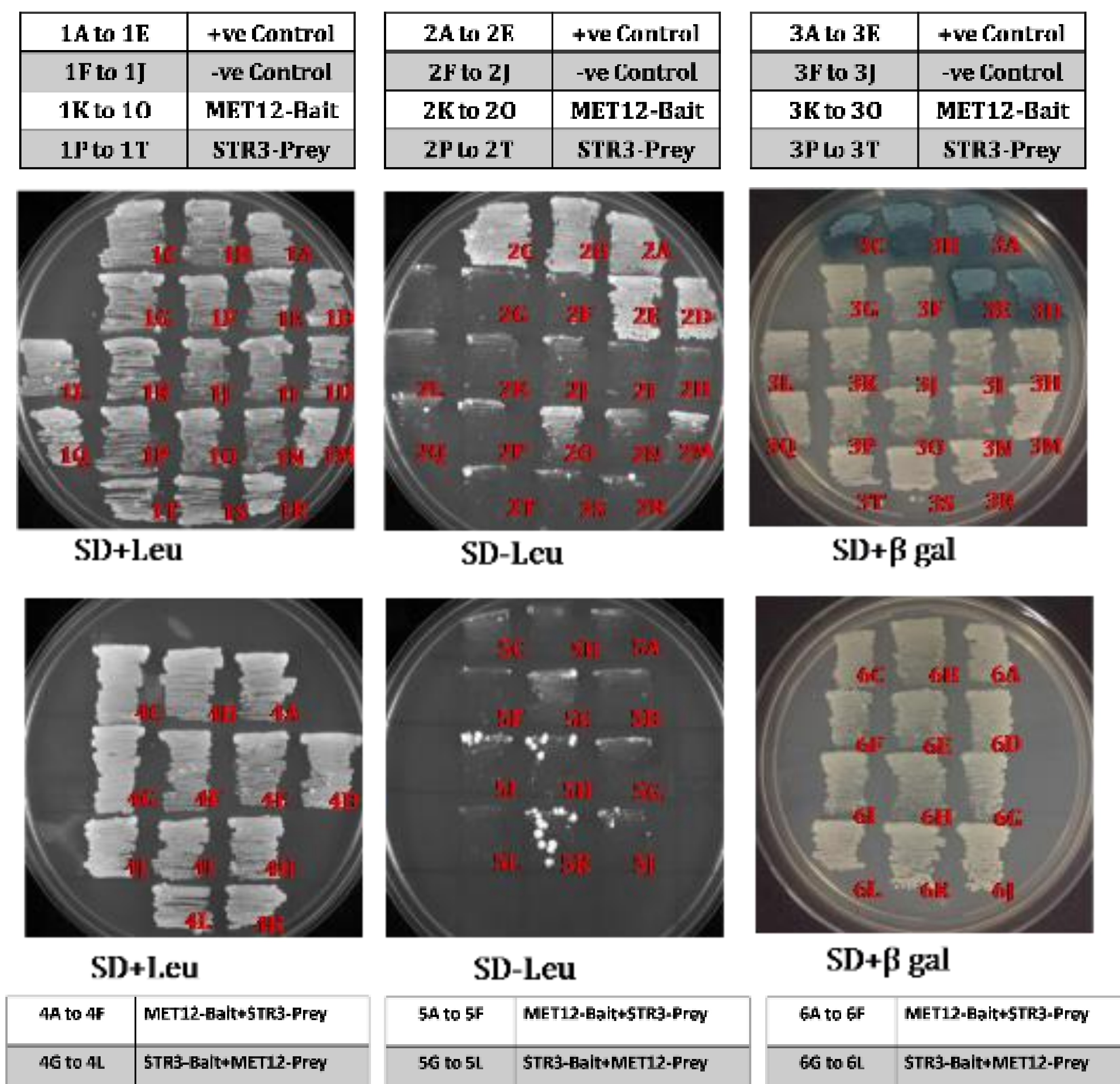


Figure 3.15: Yeast two hybrid assay to check physical interaction between *MET12* and *STR3*

3.10 Summary and Conclusions –

In this study, we attempted to understand the regulation of *STR3* which seems to be an important regulatory node in sulphur metabolism.

Phylogenetic footprinting data revealed the presence of several conserved motifs in *STR3* promoter region. Further studies for examining the *STR3* regulation were done using STR3 promoter-GFP reporter fusions. Strong repression of *STR3* was achieved both by methionine and cysteine (but not by glutathione which is known to be a mild repressor).

Transcription factor deletion experiments demonstrated a drastic decrease in *STR3* expression under sulphur de-repressed conditions for *gcn4Δ* background among the non-sulphur related transcription factors suggesting possible role of *GCN4* for complete de-repression of *STR3*. Among the sulphur related transcription factors, both *cbf1*Δ and *met31*Δ cause a mild decrease in *STR3* expression on the contrary *met28*Δ leads to mild increase of *STR3* levels under sulphur de-repressed conditions. Even more, *met31*Δ leads to decline in extent of repression while using methionine as sulphur source indicating a major role of *MET31* in regulation of STR3 by sulphur sources.

From deletion analysis of *STR3* promoter, we identified that Motif A (putative *UME6*) and Motif B (putative *MET31/32*) are not playing major role in *STR3* expression. Further the deletion of MOTIF C (putative *GCN4* motif) suggested a possible role of *GCN4* for complete de-repression of *STR3*. Mutational studies of individual *MET31/32* motifs present in *STR3* promoter could not depict any role of these motifs individually.

STR3 also did not appear to be upregulated by elevated levels of intracellular cystine.

Our data shows that *STR3* gene is not regulated by nitrogen source despite the presence of conserved region for the binding of nitrogen pathway regulators (*UME6*,*GLN3*).

While deciphering the link between *MET12* and *STR3*, we didn’t find any transcriptional block at *STR3* step in *met12*Δ*met15*Δ and also didn’t observe physical interaction between *MET12* and *STR3* in yeast two hybrid assay.

The reason why *STR3* promoter has so many conserved regions and what role exactly they are playing is still unclear.

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